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Published in:
Journal of Biotechnology

DOI:
10.1016/j.jbiotec.2009.01.020

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Kelly, R. M., Dijkhuizen, L., & Leemhuis, H. (2009). Starch and alpha-glucan acting enzymes, modulating their properties by directed evolution. *Journal of Biotechnology*, *140*(3-4), 184-193. https://doi.org/10.1016/j.jbiotec.2009.01.020

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Journal of Biotechnology 140 (2009) 184–193

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Review

Starch and α-glucan acting enzymes, modulating their properties by directed evolution

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ARTICLE INFO

Article history:
Received 12 October 2008
Accepted 29 January 2009

Keywords:
Glucansucrase
Glucanotransferase
Screening
Amylase
Selection
Protein engineering

ABSTRACT

Starch is the major food reserve in plants and forms a large part of the daily calorie intake in the human diet. Industrially, starch has become a major raw material in the production of various products including bio-ethanol, coating and anti-staling agents. The complexity and diversity of these starch based industries and the demand for high quality end products through extensive starch processing, can only be met through the use of a broad range of starch and α-glucan modifying enzymes. The economic importance of these enzymes is such that the starch industry has grown to be the largest market for enzymes after the detergent industry. However, as the starch based industries expand and develop the demand for more efficient enzymes leading to lower production cost and higher quality products increases. This in turn stimulates interest in modifying the properties of existing starch and α-glucan acting enzymes through a variety of molecular evolution strategies. Within this review we examine and discuss the directed evolution strategies applied in the modulation of specific properties of starch and α-glucan acting enzymes and highlight the recent developments in the field of directed evolution techniques which are likely to be implemented in the future engineering of these enzymes.

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1. Carbohydrates/α-glucans

In green plants starch is formed as a product of photosynthesis, acting as a major chemical repository for solar energy. Starch is primarily comprised of two different glucose polymers, amylose and amylopectin. Amylose which makes up 15–25% of starch is comprised of long linear chains of glucose residues linked via α-(1,4)-glycosidic bonds, with approximately 0.1% of α-(1,6)-glycosidic branch points (Kossmann and Lloyd, 2000) (Figs. 1 and 2). In contrast, amylopectin consists mainly of α-(1,4)-linked glucose units but with 5–6% of α-(1,6)-glycosidic branch points. Amylopectin, one of the largest polymers found in nature, has a molecular weight between 10^7 and 10^8 Da, while amylose has a lower molecular weight of 5×10^5 to 10^6 Da (Buléon et al., 1998). Within the amyloplasts of seeds, tubers and roots starch is synthesized and accumulates as water-in-soluble granules for long-term storage (van der Maarel et al., 2002). Starch granules vary in both size (1–100 μm) and shape depending on the biological origin. These granules are comprised of alternating semi-crystalline and amorphous layers. The semi-crystalline layers are built of double

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0168-1656/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2009.01.020

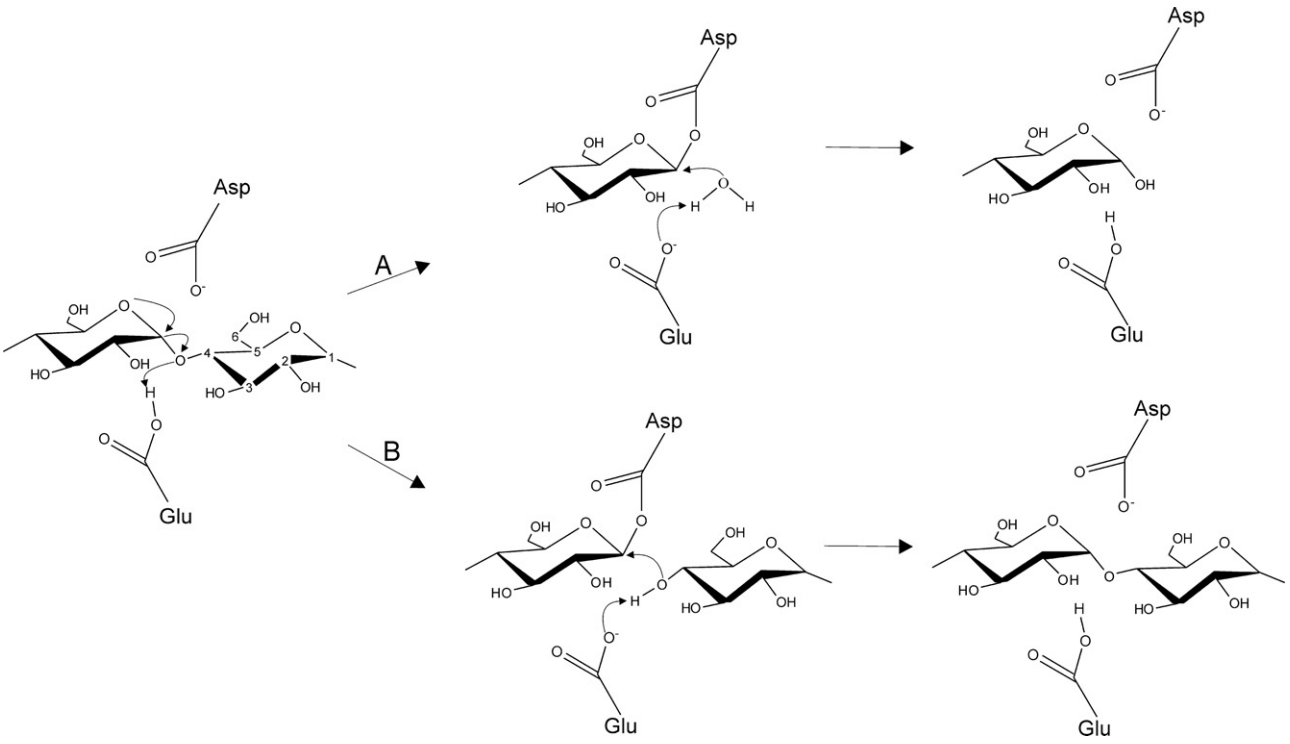

Fig. 1. The α-retaining double displacement bond mechanism employed by enzymes of the α-amylase clan GH-H. The glycoside ring nomenclature is incorporated in the substrate. The type of acceptor substrate utilised in the second half of the reaction determines enzyme reaction specificity; (A) a water molecule for hydrolases and (B) a hydroxyl group of a saccharide for the glucanotransferases.

helices formed by short amylopectin branches. The amorphous layers are comprised mainly of amylose and non-ordered amylopectin branches (Buléon et al., 1998; Kossmann and Lloyd, 2000). In mammals glycogen is the storage form of glucose, analogous to starch in plants. Structurally glycogen is similar to amylopectin but with a higher degree of α-(1,6) branches at approximately 9% (Melendez et al., 1998).

Apart from acting as a key energy reservoir, carbohydrates also have other essential roles to play in all kingdoms of life. The α-glucans, commonly produced by bacteria including *Streptococcus* and *Lactobacillus* from sucrose, are believed to play an essential role in protecting these microbes against desiccation, osmotic stress, antibiotics and toxic compounds (van Hijum et al., 2006). Structurally, these complex homopolymers consist of glucose residues linked mainly via α-(1,4)-(reuteran) (Kralj et al., 2002), α-(1,6)-(dextran) (Kralj et al., 2005), α-(1,6)- and α-(1,3)-(alternan) (Balakrishnan et al., 2000) and α-(1,3) (mutan) glycosidic bonds (Kralj et al., 2004; van Hijum et al., 2006) (Fig. 2).

The complexity of these starch and glucan structures is mirrored by the requirement of organisms to produce an array of enzymes for their synthesis, breakdown and modification. The majority of these starch and glucan acting enzymes belong to the α-amylase clan GH-H consisting of glycoside hydrolase families 13, 70 and 77 (http://www.cazy.org/) though they are also found among the GH families 14, 15, 31 and 57 (Coutinho and Henrissat, 1999a; Coutinho and Henrissat, 1999b; Zona et al., 2004; Stam et al., 2006; Janecek et al., 2007). These enzymes either transglycosylate or hydrolyze α-glycosidic linkages (Fig. 1). The GH13, 31, 57, 70 and 77 enzymes employ a retaining mechanism with the initial formation of a covalent glycosyl intermediate prior to forming α-anomeric products (Uitdehaag et al., 1999) (Fig. 1). The GH14 and 15 enzymes, in contrast, yield β-anomeric products via an inverting mechanism (McCarter and Withers, 1994; Mizuno et al., 2004). The nature of the acceptor molecule used by these enzymes determines their reaction specificity; the hydroxyl group of a saccharide in the case of glucanotransferases and a water molecule for hydrolases (Fig. 1). Even amongst these glucanotransferases and hydrolases the substrate- and product specificity may vary considerably. Isoamylases for example hydrolyze α-(1,6) bonds while α-glucosidases hydrolyze α-(1,4) bonds (Fig. 2). The cyclodextrin glucanotransferases (CGTases) disproportionate α-(1,4) bonds to yield cyclic α-(1,4)-oligosaccharides (cyclodextrins) while branching enzymes cleave α-(1,4) bonds before reattachment of the glucan chain by an α-(1,6) bond (MacGregor et al., 2001; Thiemann et al., 2006) (Fig. 2). The glucansucrases of GH70 form linear as well as branched α-glucan polymers including reuteran, dextran, alternan and mutan from sucrose (Potocki-Veronese et al., 2005; van Hijum et al., 2006) (Fig. 2).

Many of these starch and glucan acting enzymes are of great commercial value in the modification and conversion of starch due to their unique capacity to catalyze reactions at high velocity and specificity under environmentally friendly conditions without the addition of expensive activated sugars like ADP- and UDP-glucose (Buchholz and Seibel, 2008). The well documented α-amylases of GH13 play a vital role in the sugar producing industry (Nielsen and Borchert, 2000; Plou et al., 2007). Starch from wheat, maize and tapioca is hydrolyzed to maltodextrins by thermostable α-amylases like that from *Bacillus licheniformis* and then saccharified to glucose by glucoamylase (Crabb and Shetty, 1999) (Fig. 2). Several of the starch acting enzymes are applied in diverse industrial applications such as production of bio-ethanol, surface sizing, laundry, anti-staling agents in baking, coating agents in the paper industry, pulp processing and detergent industry (van der Maarel et al., 2002; Turner et al., 2007; Buchholz and Seibel, 2008). Despite the ever increasing number of starch modifying enzymes isolated from hyperthermophilic bacteria and archaea, naturally occurring starch acting enzymes are usually not optimally suited to the adverse conditions encountered in industrial applications, or have limited shelf lives (Bertoldo and Antranikian, 2002). Many of the industrial bioprocessing conditions are of elevated temperature and extreme

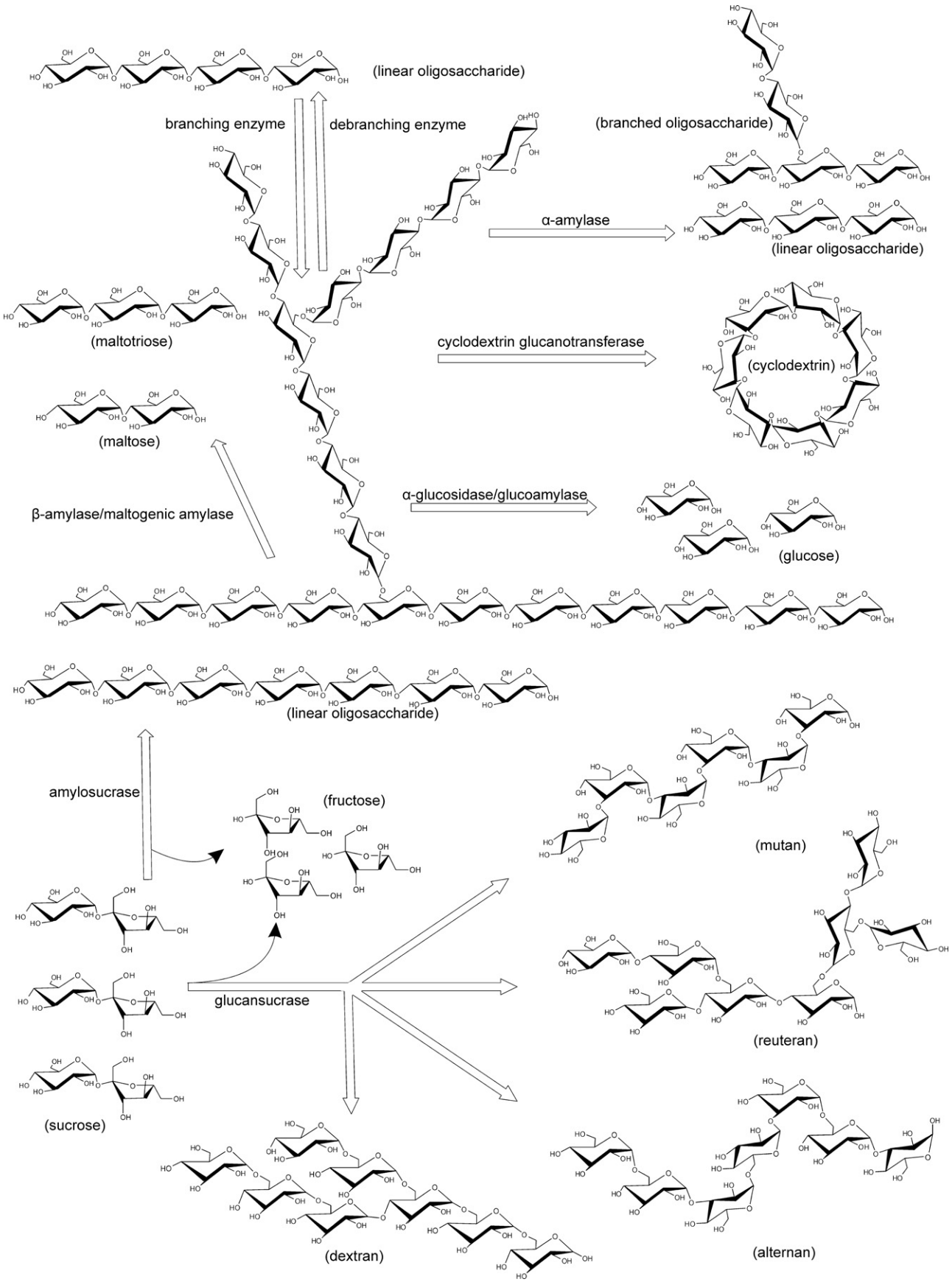

Fig. 2. Schematic representation of the action of starch and α-glucan acting enzymes.

pH to reduce the risk of microbial contamination, enhance substrate and product solubility and increase process speed (Crabb and Shetty, 1999). Various other enzyme properties including reaction rates, product specificity, product feedback inhibition, overall substrate conversion and product yields are also of major importance during the selection of amylolytic enzymes for industrial applications.

The incompatibility encountered between industrial requirements and native environment for these starch and α-glucan modifying enzymes results in suboptimal enzymatic performance in many applications. Although it may be possible to adapt the industrial process to more favorable conditions for the enzyme, ultimately it is more financially feasible to adapt the enzyme to the prevailing process conditions. To improve the performance characteristics of the enzyme of interest, directed evolution strategies are frequently applied. In this review we examine and discuss the effectiveness of these molecular evolutionary strategies applied in the modulation of starch and α-glucan modifying enzymes (summarized in Table 1).

2. Directed evolution techniques

Since the 1990s an array of directed evolution techniques have been developed allowing for engineering of desired enzyme and protein properties within restricted time frames. This evolutionary approach is based on Darwinian principles whereby random genetic diversity is generated in genes, followed by screening and selection for variants with the desired improvements (Fig. 3). The gene(s) encoding the improved variant(s) may then act as parent for multiple iterations of this two-step process enhancing the desired properties of the enzyme or protein further. Prior to the emergence of directed evolution techniques scientists relied on rational protein engineering for the improvement of enzymes, which required extensive information of the structure-function relationship of the enzyme in question. The directed evolution approach, in contrast, requires little or no information on protein structure and mechanism. In the laboratory, diversity in the gene of interest may be created using a variety of techniques. Genetic diversity is generated by deliberately disturbing the copying of DNA sequence during the Polymerase Chain Reaction (PCR) cycles, e.g. the DNA is amplified under sub-optimal conditions such as the addition of Mn^{2+} ions in the PCR which triggers misincorporation of nucleotides throughout the gene sequence (Fig. 3) (Neylon, 2004; Kelly et al., 2008a). In contrast to the error-prone PCR (epPCR) method, synthesis of partially or fully randomized oligonucleotides provides a means to generate diversity at specific locations in a gene (Fig. 3). Site-saturated mutagenesis is one of the occasional instances where a high resolution structure of the target protein is truly beneficial in directed evolution experiments. This technique replaces a specific residue of the protein with the 19 other amino acids, and has been successfully applied in numerous protein engineering studies (Park et al., 2005; Parikh and Matsumura, 2005; Reetz et al., 2006; Kelly et al., 2007; Di et al., 2007).

In addition to the non-recombinative methods mentioned, a widely used approach for the generation of gene library diversity is the use of homologous recombination of closely related genes in a process called DNA shuffling (Stemmer, 1994b). This evolutionary method involves the controlled fragmentation of source DNA either by DNAase I (Stemmer, 1994a; Zhao and Arnold, 1997) or common restriction enzymes prior to a primer-less PCR gene assembly (Fig. 3) (Kikuchi et al., 1999; Kaper et al., 2002; Rosic et al., 2007). A number of additional, more intricate techniques, although applied on a less frequent basis, have also been devised for the creation of libraries, such as ITCHY (incremental truncation for the creation of hybrid enzymes) (Ostermeier and Lutz, 2003), RACHITT (random chimeragenesis on transient templates) (Coco et al., 2001) and random circular permutation (Qian et al., 2007); see the indicated references for details. While the most suitable technique for generating genetic diversity is largely dependent on the preference of the scientist, DNA shuffling has been shown to be extremely effective in generating strongly improved variants in combination with epPCR. By combining both methods it is possible to bring together advantageous mutations while removing deleterious mutations in a technique similar to sexual recombination (Table 1) (Miyazaki et al., 2000; Matsumura and Ellington, 2001; van Loo et al., 2004; Aharoni et al., 2005; Luginbuhl et al., 2006).

3. Screening and selection

While the development of gene libraries with suitable sequence diversity is an essential step, the design of a suitable selection or screening for identification of variants with improved properties is paramount. A diverse range of selection and screening technologies are available to scientists in an effort to isolate variants from mutant libraries with the desired properties (Williams et al., 2004; Bershtein and Tawfik, 2008). A relatively cheap and rapid screening method involves the direct link between survival and growth of the host organism on agar plate for improved or acquired enzyme function (Fig. 3) (Bosma et al., 2002). Excretion of enzyme variants into the surrounding solid media containing substrate also allows for the rapid identification of improved variants by enlarged halo formation compared to wild-type enzyme (Shim et al., 2004). An alternative to this relatively simple plate assay is the screening of each individual member of the library in 96/384/1536-well microtitre plates (Fig. 3). The microtitre plate screening assay is based upon lysis of cell culture in individual wells prior to addition of substrate dissolved in a suitable buffer. The reaction progress of samples is often monitored directly by incubation of cell lysate with selected compounds for the detection of specific products through colourimetric analysis in a microtitre plate reader. Such a screening method allows for selection of variants with improved catalytic rates, altered substrate specificity, thermo-/solvent stability. An advantage of microtiter plate based screenings is that they generally have a broader dynamic range which allows for detection of relatively low improvements in the desired enzyme function compared to the colony screening assays.

Although the screening and selection methods mentioned above have proved highly successful in identification of improved variants, the microtitre plate assay remains tedious to work with, restricts the numbers of mutants that can be examined and is generally expensive as it requires large amounts of consumables and substrates. The development, however, of *in vitro* protein evolution technologies holds great potential in the screening of large number of variants. Methods such as ribosome display and mRNA display, which rely on holding together the transcribed protein and the encoding mRNA molecule to link the geno- and phenotype, have proven to be very powerful in the selection of strong binding proteins from libraries with up to $\sim 10^{12}$ members (Jermutus et al., 2001; Seelig and Szostak, 2007). For the selection of enzymes, however, other methods are required to link the "phenotype" (the products of the reaction catalyzed by the enzyme) back to the genotype. *In vitro* compartmentalization provides a fully *in vitro* platform for ultra high-throughput ($>10^7$ day^{-1}) screening of enzymes (Tawfik and Griffiths, 1998; Leemhuis et al., 2005). It involves droplets with a size of only a few femto-liter that possess a single gene and a transcription/translation mixture that produces multiple copies of the encoded protein within a droplet (Fig. 3). The droplets are made by emulsifying a water phase (containing the genes, transcription/translation mixture and substrate) in oil

Table 1
Summary of improvements of evolved starch and α-glucan acting enzymes.

Target protein	Method	Library size	Screening system (improvement)	References
α-Amylase	Shuffling	21,000	Hydrolytic act. at high temp. in absence of Ca^{2+} (40-fold longer half-life at 90 °C and pH 4.5)	Richardson et al. (2002)
α-Amylase	epPCR/shuffling	17,200	Hydrolytic act. at elevated pH's (5-fold higher act. at pH 10)	Bessler et al. (2003)
α-Amylase	Combined saturated mutagenesis	50,000	Binding of phage particles at low pH (2.6-fold inc. pH 4.5/7.5 ratio of starch hydrolysis)	Verhaert et al. (2002)
α-Amylase	epPCR/shuffling	53,000	Hydrolytic act. (20-fold higher specific act.)	Wong et al. (2004)
α-Glucan phosphorylase	epPCR	25,000	Phosphorylase act.[a] (retention of catalytic act. after heat treatment at 60 °C for 2 h)	Yanase et al. (2005)
Amylomaltase	epPCR/saturated mutagenesis	11,384	Disproportionation and hydrolytic activity (increased transglycosylation/hydrolytic activity 140-fold)	Kaper et al. (unpublished data)
Amylomaltase	epPCR/saturated mutagenesis	3,000	Assayed for cyclization and hydrolytic act. (10-fold lower hydrolytic/cyclization[b])	Fujii et al. (2005)
Amylosucrase	epPCR/shuffling	25,000	Transglycosylation and hydrolytic act. (2-fold higher catalytic efficiency)	van der Veen et al. (2004)
Amylosucrase	epPCR	67,000	Transglycosylation act.[a] (half-life inc. 10-fold at 50 °C)	Emond et al. (2008)
Cyclodextrin glucanotransferase	epPCR/saturated mutagenesis	12,288	β-Cyclization act. in the presence of inhibitor (6700-fold inc. in IC_{50})	Kelly et al. (2008a)
Cyclodextrin glucanotransferase	Saturated mutagenesis	1,340	β-Cyclization and hydrolytic act. (increased hydrolysis/cyclization ratio 4055-fold)	Kelly et al. (2007)
Cyclodextrin glucanotransferase	epPCR	3,000	β-Cyclization and hydrolytic act. (15-fold higher hydrolytic act.)	Shim et al. (2004)
Cyclodextrin glucanotransferse	epPCR	32,000	Hydrolytic act. (90-fold higher hydrolytic act.)	Leemhuis et al. (2003)
Cyclodextrin glucanotransferase	epPCR/saturated mutagenesis	12,600	β-Cyclization and hydrolytic act. (8.3-fold[c] higher ratio of β-cyclization/hydrolysis)	Kelly et al. (2008b)
Glucansucrase	Mutagenesis by ultrasoft X-rays	500	Transglycosylation act. (2.3-fold higher glucansucrase act.)	Kang et al. (2003)
Glucansucrase	Saturated mutagenesis	2,000	Transglycosylation act. and polymer solubility (synthesis of polymer with mainly α-(1,3) glycosidic linkage)	Hellmuth et al. (2008)
Glucoamylase	epPCR/Shuffling	42,000	Hydrolytic act.[a] (5.1 kJ/mol in free energy of thermo-inactivation)	Wang et al. (2006)
Glycosynthase	epPCR/saturated mutagenesis	20,900	Transglycosylation act. (27-fold higher catalytic efficiency)	Kim et al. (2004)
Maltogenic amylase	Shuffling	4,000	Hydrolytic act.[a] (15 °C inc. optimal reaction temp.)	Kim et al. (2003)
Maltogenic amylase	Shuffling	28,000	Hydrolytic act.[a] (10 °C inc. optimal reaction temp.)	Tang et al. (2006)
Maltogenic amylase	epPCR/shuffling	113,000	Hydrolytic act. at lowered pH (10 °C inc. melting temp. at pH 4)	Jones et al. (2008)

Abbreviations: act., activity; inc., increase: temp., temperature; epPCR, error-prone PCR.
[a] Assay for native activity after incubation at elevated temperatures.
[b] 30% increase in final yield of cycloamylose.
[c] 81% increase in the final yield of β-cyclodextrin from product profiles from starch activity.

(Tawfik and Griffiths, 1998; Taly et al., 2007). Only droplets possessing a gene encoding a functional enzyme will convert substrate into products, which may be detected via fluorescence, enabling screening of large libraries ($>10^7\ h^{-1}$) by fluorescence-activated cell sorting (FACS), as successfully shown for the evolution of a β-galactosidase (Mastrobattista et al., 2005). In an effort to further extent the power of screening technologies, scientists are working to try and merge the *in vitro* compartmentalization approach with microfluidics technology. The most recent papers describe the construction of pico-liter reactors (basically small droplets) using microfluidic devices carrying single (bacterial) cells (Huebner et al., 2007; Courtois et al., 2008). This approach is similar to a microtiter plate screen, but then with "micro-reactors" at a 10^6-times smaller volume. This procedure was successfully applied to investigate the enzyme kinetics of a wild-type and mutant alkaline phosphatase (Huebner et al., 2008). The advantages of microfluidics include tight control over the life-time of the micro-reactors, the addition of substrates and or quenchers at the desired time followed by analysis of the contents. Such an approach will expand the evolutionary screening boundaries of protein engineering and allow for a greater understanding of the specific cellular function of individual carbohydrate modifying enzymes.

4. Laboratory evolution of α-glucan acting enzymes

4.1. Evolving enzyme (thermo) stability

The stability of biocatalysts is often a limiting factor in the selection of enzymes for industrial applications due to the elevated temperatures or extreme pH of many biotechnological processes. The stability properties of enzymes may be improved by various methods such as site-directed mutagenesis (Leemhuis et al., 2004; Liu et al., 2008), immobilization techniques (Grazu et al., 2005) or directed evolution approaches (Eijsink et al., 2005). The corn wet milling industry for example applies temperatures of up to 105 °C for the liquefaction of semi-purified starch using *B. licheniformis* α-amylase (Fig. 2) (Richardson et al., 2002). The high temperature, however, requires the addition of calcium ions to stabilise the α-amylase enzyme. The Ca^{2+} ions, however, inhibit the glucose isomerase enzyme used in the final step of the process, for the conversion of glucose to high fructose corn syrup and in addition may lead to the formation of inorganic precipitates which have deleterious effects on fermentation and downstream processing. Removal of these metal ions is both costly and time consuming to the overall industrial process. The use of stable, functional α-amylases in the

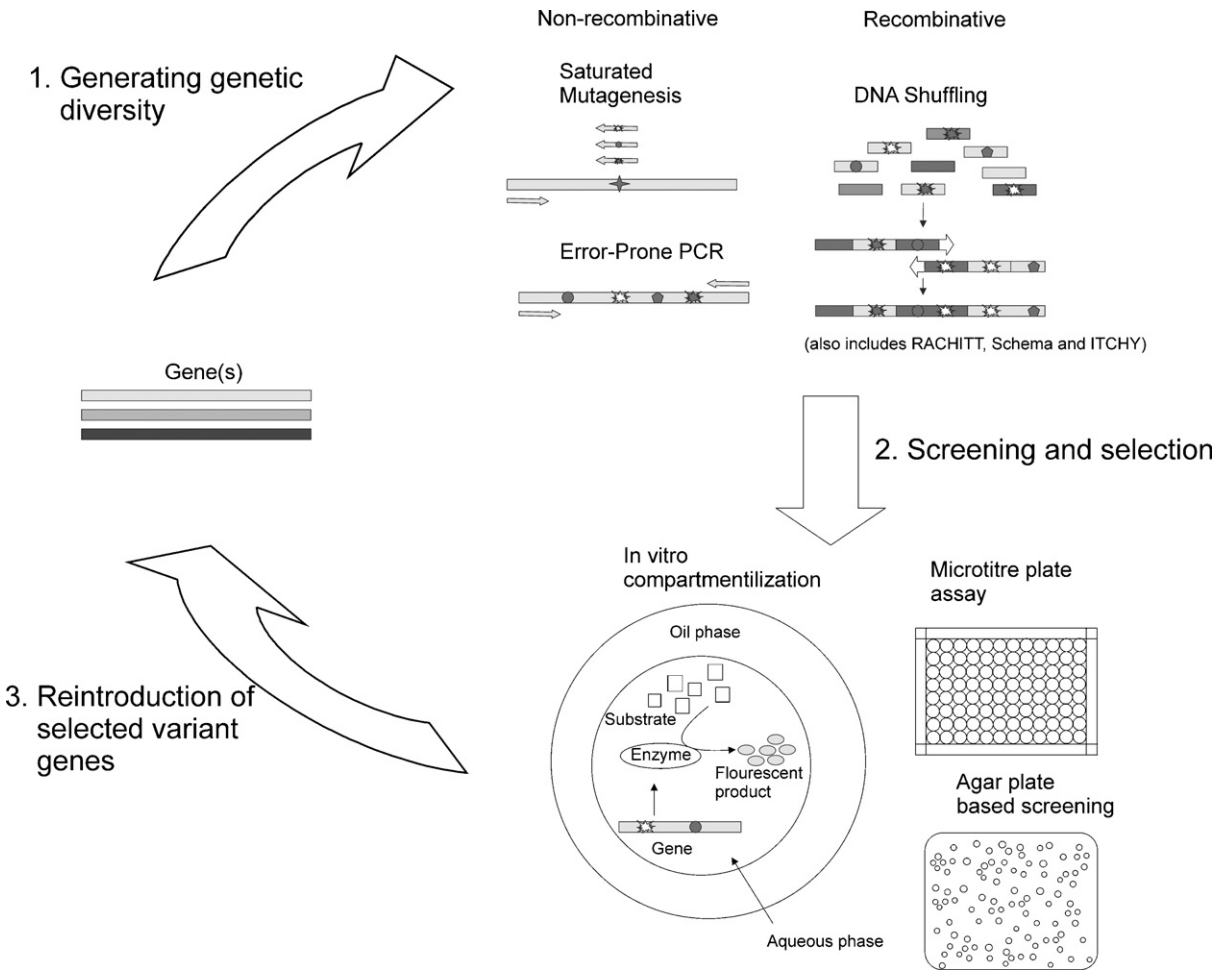

Fig. 3. Schematic representation of the directed evolution process. Step 1. Creation of genetic diversity via recombinative and/or non-recombinative methods. Step 2. Screening for desirable proteins by *in vivo* (bacteria, fungi) or *in vitro* technologies using affinity, colorimetric or growth selection assays. Step 3. Further rounds of directed evolution are carried out using the first generation gene as parent.

absence of Ca^{2+} ions at high temperatures would be highly favored. Isolation of starch modifying enzymes from highly hyperthermophilic bacteria and archaea may provide a valuable alternative to such calcium dependent enzymes. While these enzymes may initially be suboptimal for application in industry in terms of poor reaction specificity or low protein expression, the high thermostability properties offer protein engineers a genetic template from which to create a highly stable enzyme with the desired properties. Richardson et al. (2002) applied activity and sequence based screening approaches to single and multi-organism DNA libraries from various environments to identify three different α-amylases with one or more aspects of the necessary phenotype: temperature stability, pH optimum, lowered reliance on calcium ions and/or enzyme rate compatible with large scale corn wet milling process conditions. The genes encoding these three enzymes were used as parental sequences for DNA shuffling in order to combine the best aspects of the three enzyme phenotypes (Fig. 3). The two fittest chimeric α-amylases found by high throughput screening had 40-fold longer half-life activity in the absence of calcium ions, at 90 °C and pH 4.5 compared to the most stable wild-type parent.

Alternatively, directed evolution strategies may be applied to enhance the thermostability of enzymes with pre-existing favorable enzymatic properties in the absence of calcium ions. To our knowledge this strategy has not yet been described for an α-amylase, but the principle has been demonstrated for the xylanase from *Cellvibrio japonicus*. Three rounds of error-prone PCR raised the inactivation temperature of this enzyme in the absence of calcium by 10 °C, without affecting its catalytic potential (Andrews et al., 2004). Glucoamylase is used industrially to catalyze the release of β-D-glucose from the non-reducing ends of starch or oligosaccharides (Fig. 2). Initially starch is treated with α-amylase at 105 °C, before the reaction mix is cooled to 60 °C to accommodate the lower thermostability of glucoamylase. In order to avoid this cooling step and increase the activity of the enzyme at higher temperatures, random mutagenesis was applied to a glucoamylase from *Aspergillus niger* (Wang et al., 2006). Library variants were screened using a starch-plate based assay followed by random recombination of selected mutations yielding a triple mutant with enhanced thermostability. These three mutations were then combined with previously constructed site directed mutants to create the most thermostable *A. niger* glucoamylase mutant characterized to date, with a 5.1 kJ/mol increase in free energy of thermal inactivation. Specific activities and catalytic efficiencies remained similar between the variant and wild-type.

DNA shuffling has also proved highly successful in improving the thermostability of maltogenic amylases (Fig. 3) (Kim et al., 2003; Tang et al., 2006). These enzymes are used in industry for the solubilization of high-value compounds by the addition of sugar residues and the synthesis of novel carbohydrates (Park et al., 1998). Three rounds of DNA shuffling of *Bacillus thermalkalophilus* ET2 maltogenic amylase followed by recombination of selected mutations yielded variants with optimal reaction temperatures 10 °C higher

than wild-type enzyme. One of these variants carrying 7 mutations displayed a 20-fold longer activity half-life than wild-type at 78 °C (Tang et al., 2006). In a separate study, four rounds of DNA shuffling and subsequent recombination of mutations, produced a maltogenic amylase variant from *Thermus* sp. strain IM6051 with a 15 °C increase in optimum temperature compared to wild-type (Kim et al., 2003). The activity half-life of this variant was 172 min at 80 °C, while the wild-type was completely inactivated at this temperature in less than 1 min.

The thermostability of a potato type L α-glucan phosphorylase which catalyzes the reversible phosphorylation of α-1,4 glucan, was enhanced by random mutagenesis (Yanase et al., 2005). This enzyme plays an important role in the cellular metabolism of the reserve polysaccharides starch and glycogen. Three variants with increased thermostability were selected by transferring colonies from agar plates to a nylon membrane, which was subjected to heat treatment and examined for remaining α-glucan phosphorylase activity. All three improved variants carried a single mutation and the combination of these single mutations yielded a triple mutant (F39L, N135S, T706I) retaining 50% of its original activity after heat treatment at 65 °C for 20 min. Wild-type enzyme lost all activity after incubation at the same temperature for the same amount of time.

4.2. Evolving the pH optimum of enzymes

The use of starch modifying enzymes in industrial applications at specific pH often necessitates the adaptation of the enzyme to the prevailing process conditions. The α-amylase from *Bacillus amyloliquefaciens*, for example, has optimal activity at pH 6. In order to create a highly active enzyme at alkaline pH for the detergent industry, the *B. amyloliquefaciens* α-amylase and two variants carrying single point mutations were optimized by directed evolution (Bessler et al., 2003). Screening of an epPCR library for amylase activity at pH 7 and pH 10, yielded 26 variants with higher activity. Sixteen of the selected variants were then randomly recombined by DNA shuffling and screened for increased activity at pH 10. The best mutant displayed a 5-fold higher activity at pH 10 than wild-type.

In an effort to improve the performance of a maltogenic amylase (Novamyl) as an antistaling agent for breads made at low pH recipies, two epPCR libraries of Novamyl were constructed (Jones et al., 2008). These libraries were screened for improved thermal stability at 80 °C and activity at pH 4.3 for 25 min. A triple mutant significantly outperformed wild-type's antistaling activity in bread made at pH 4.3 and exhibited a 10 °C increase in melting temperature at pH 4 compared to wild-type.

With the availability of high resolution 3D structures, site directed mutagenesis may also be applied to alter the optimum pH of an enzyme (Fig. 3). This method alleviates the time and cost spent on screening libraries for improved variants. Saturated mutagenesis of the Cβ4 starch-binding domain of the α-amylase from *B. licheniformis* followed by selection for starch binding at low pH yielded a double mutant with an improved starch hydrolysis ratio at pH 4.5/7.5 of 0.13 compared to 0.05 of wild-type (Verhaert et al., 2002). Saturated mutagenesis also proved successful in altering the pH optimum of a soybean β-amylase. The hydrogen bond networks around the catalytic base residue, E380, of the enzyme were removed by point mutations (Hirata et al., 2004), raising the optimum from pH 5.4 to a more neutral pH range between 6 and 6.6. However, the catalytic rate was severely affected for all three mutants, highlighting the drawback of the site-directed mutagenesis approach. A directed evolution approach, in contrast, including screening and selection steps, ensures the selection of variants with sufficient activity at the chosen pH.

4.3. Evolving product and reaction specificity

Despite the favorable protein characteristics of high thermostability and activity under a broad pH range, an enzyme's use as an industrial tool for the synthesis, modification, or degradation of starch and related α-glucan polymers may be limited due to low catalytic efficiency, its catalysis of non-desired side reactions, or low expression yields of the enzyme. The amylosucrase from *Neisseria polysaccharea* for example synthesizes an amylose-like glucan from sucrose (Fig. 2). The catalytic rate, however, is low (k_{cat} = 1 s^{-1}) and sucrose isomers are formed as by-products. The catalytic efficiency of the amylosucrase was improved approximately 4-fold through epPCR and gene shuffling followed by high-throughput screening (van der Veen et al., 2004; van der Veen et al., 2006). In addition, the selected variants were found capable of α-glucan polymer formation at lower sucrose concentrations (10 mM) than the wild-type enzyme.

In an attempt to increase the amount of barley α-amylase isozyme 2 activity produced by *Saccharomyces cervisiae*, three rounds of epPCR mutagenesis followed by gene shuffling were applied to the amylase gene (Wong et al., 2004). 53,000 variants were screened for halo formation on starch agar plates followed by a high-throughput liquid assay using dye labelled starch as substrate. A selected mutant enzyme carrying five mutations displayed 20 times greater specific activity than wild-type enzyme. In addition, the expression level of the selected mutant was 50-fold higher, thus increasing the total amount of α-amylase activity obtained 1000-fold. This experiment demonstrated that directed evolution is a powerful procedure to enhance the absolute amount of activity, which is a crucial parameter in industry.

The enzymatic capabilities of glucansucrase enzymes of family GH70 have also been engineered by directed evolution (Kang et al., 2003; Nam et al., 2008; Hellmuth et al., 2008). Glucansucrases, as mentioned earlier, are extracellular sucrases that synthesize complex α-glucans from sucrose (Fig. 2). Genetic variation was generated by exposing the glucansucrase gene of *Leuconostoc mesenteroides* B-742CB to ultrasoft X-rays prior to transformation of *Escherichia coli*. Approximately, 500 colonies were found forming mucous dextran on agar plates containing sucrose. Subsequent analysis of these variants yielded a mutant enzyme with 2.3 times higher glucansucrase activity compared to the parent clone (Kang et al., 2003). The glucan product formed by this mutant enzyme contained a much higher degree of branching (2.7 times) compared to wild-type. In an effort to engineer the reaction specificity of the glucansucrase GTFR from *Streptococcus oralis* Hellmuth et al. (2008) randomized the conserved motif near the transition state stabiliser via site saturated mutagenesis. Screening yielded a triple mutant, R624G/V630I/D717A, forming predominantly α-(1,3)-glycosidic linkages (e.g. a mutan like polymer), whereas the wild-type enzyme produces mainly α-(1,6)-linkages. Another mutant (S628D) isolated formed 25-times more isomaltose when glucose was added as acceptor substrate (Hellmuth et al., 2008).

Directed evolution is also a powerful technique to lower or purge enzyme inhibitory effects. Many enzymes acting on starch for example are strongly inhibited by the small molecule acarbose. By applying epPCR mutagenesis acarbose insensitive CGTase variants were generated that retained native β-cyclodextrin forming activity (Kelly et al., 2008a). Three single mutants were identified (K232E, F283L, and A230V) that raised IC_{50} values for acarbose 3 to 4 orders of magnitude (Kelly et al., 2008a). The same strategy can be applied to lower or remove product inhibition, by screening mutant libraries for high product yields and or efficient substrate conversion in the presence of products.

Alternatively, certain evolution studies have focused on interchanging enzymes reaction specificity by enhancing side reaction rates and decreasing the primary activity of the enzyme. Both epPCR

mutagenesis and combined saturated mutagenesis were separately applied to investigate the evolutionary input required to interchange the primary and side reaction specificity of a cyclodextrin glucanotransferase (Kelly et al., 2007). CGTases primarily catalyze a transglycosylation reaction for the formation of circular saccharides (cyclodextrins) from starch but also display α-amylase like activity as a minor side reaction, hydrolyzing starch into short saccharides (Fig. 2). The generation of a combined saturated mutagenesis library at the acceptor subsites of *Thermoanaerobacterium thermosulfurigenes* EM1 CGTase followed by screening and selection of variants with a high hydrolysis and low cyclization rates, yielded a triple mutant, A231V/F260W/F184Q. This mutant displayed the highest hydrolytic rate (k_{cat} of 730 s^{-1}) ever recorded for a CGTase, while cyclodextrin production by the enzyme was virtually abolished. Screening of a much larger epPCR generated library, yielded far less effective mutants. A similar approach yielded a *Bacillus circulans* 251 CGTase variant with a 90-fold increase in hydrolytic activity (Leemhuis et al., 2003). A combination of epPCR and site directed mutagenesis were applied in an effort to generate CGTase variants from alkalophilic *Bacillus* sp. I-5 with improved properties as an antistaling enzyme (Shim et al., 2004). A triple mutant was created M234T/F259I/V591A, with a 10-fold decrease in cyclization activity and 15-fold increase in hydrolyzing activity. The CGTase triple mutant reduced the retrogradation rate of bread by as much as the commercial antistaling enzyme, Novamyl during 7-day storage at 4 °C. In addition Kaper et al. (unpublished data) recently characterized an amylomaltase variant (V246A/E224V/S274P), created via epPCR and saturated mutagenesis, with a 140-fold increase in the overall hydrolysis/transglycosylation ratio. The most effective mutations interchanging the reaction specificities of all four of these glucanotransferases were located at the acceptor subsites, highlighting the regions importance in the determination of glucanotransferase reaction specificity.

While enhancement of the hydrolytic side reaction of CGTase may have useful applications in the bread baking industry, it has a detrimental effect on the overall production of cyclodextrins. Short saccharides produced from the hydrolytic side reaction are used in the breakdown of cyclodextrins during the coupling reaction limiting final product yields. Increasing cyclodextrin yield and lowering production costs is of particular interest to food, cosmetic and pharmaceutical industries where these circular saccharides have many useful applications (Qi and Zimmermann, 2005; Loftsson and Duchene, 2007). To investigate if final cyclodextrin product yields could be increased by lowering the hydrolytic side reaction, a combination of epPCR and saturated mutagenesis was applied to Tabium CGTase (Kelly et al., 2008b). Two single mutant variants were selected S77P and W239R, lowering the enzyme's hydrolytic activity up to 15-fold with retention of wild-type cyclization rates. Both mutations were found located on the outer regions of the active site. In a similar experiment the amylomaltase (4-α-glucanotransferase) from *Thermus aquaticus* was subjected to random mutagenesis followed by saturated mutagenesis in an effort to lower the hydrolytic side reaction and increase final product yields of cyclic α-1,4 glucans (e.g. cyclodextrin like molecules consisting of 17 or more glucose monomers) (Fujii et al., 2005). The most favorable variant, Y54G, displayed the lowest hydrolytic/cyclization activity ratio which was one-tenth that of wild-type enzyme, with a 30% increase in the final yield of cycloamylose.

4.4. Glycosynthase engineering

The emergence of glycosynthase enzymes in recent years has expanded the boundaries in terms of oligosaccharide synthesis. Glycosynthases are catalytic nucleophile mutants of retaining glycosidase hydrolases (Shaikh and Withers, 2008). The resulting mutants, unlike the parent, can no longer hydrolyze glycosidic bonds and are incapable of formation of glycosyl-enzyme species that is required for transglycosylation. However, when presented with a glycosyl donors bearing a good leaving group (e.g. fluoride or dinitrophenyl) of opposite anomeric configuration to the natural substrate, these enzymes carry out efficient glycosyl transfer to appropriate acceptors. The unique transglycosylation capability of these enzymes allows for the formation of complex synthetic oligosaccharides and glycoconjugates which have considerable potential as therapeutics (Faijes and Planas, 2007). The repertoire of donors and acceptors that can be used by glycosynthases and the diversity of glycosidic linkages formed has been expanded by directed evolution. For example, two rounds of random mutagenesis (epPCR) and selection were performed on the *Agrobacterium* sp. β-glucosidase nucleophilic mutant, AbgE358G (Mayer et al., 2001; Kim et al., 2004). Two highly active mutants were obtained from transformants screened using an on-plate endocellulase coupled assay. The most effective mutant increased catalytic efficiency of the glycosynthase 27-fold compared to AbgE358G, using α-D-galactopyranosyl fluoride and 4-nitrophenyl β-D-glucopyranoside as substrates. The selected mutant also displayed an expanded repertoire of acceptable substrates such as α-D-xylopyranosyl fluoride and α-D-mannopyranosyl fluoride. Recently, a universal screening approach has been described for glycosynthases which is based on detecting the release of hydrofluoric acid via a pH indicator (methyl red) (Ben-David et al., 2008). This sensitive assay allows for screening of a large number of variants and has the potential to be utilised for any glycosynthase reaction.

5. Future prospects

Other future prospects include the directed evolution of glucansucrases and branching enzymes for the synthesis of defined polysaccharides with varying degrees of branching, glycosidic linkages, solubility, and molecular mass (Hellmuth et al., 2007; Zuccaro et al., 2008). The synthesis of 'resistant starches' for example is of great potential to the food (health) industry. These complex oligo- and polysaccharides may reduce colonic and systemic immune reactivity (Nofrarias et al., 2007) by stimulating the growth of beneficial gastrointestinal microflora (Le Leu et al., 2005). To conclude, the directed evolution of amylase and α-glucan acting enzymes complemented by rapid screening techniques holds enormous potential in both enhancing enzyme functionality in the adverse industrial conditions and developing novel natural and artificial oligo- and polysaccharides for the food, health and therapeutic based industries.

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